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Effect of media, temperature and culture conditions on the species population and antibiotic resistance of enterococci from broiler chickens*

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ABSTRACT

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Aims: The effect of media type, incubation temperature and enrichment period on the species population and antibiotic susceptibility of enterococci from poultry carcass rinsates was determined.

Methods and Results: Aliquots of rinsates, incubated in BBL Enterococcosel broth at 37°C, 42°C, or 45°C for 24 and 48 h, were inoculated onto BBL Enterococcosel and M-enterococcus agar. Presumptive positive colonies were identified to species and tested for antibiotic resistance. Significant differences ($P \leq 0{\cdot}05$) were observed for media and temperature. More *Enterococcus faecalis* were isolated from M-enterococcus media and at 37°C while more *E. faecium* were isolated from Enterococcosel agar and at 45°C. The number of antibiotic-resistant *E. faecalis* and *E. faecium* were also affected by media and temperature.

Conclusions: Culture conditions for enterococci affect the observed species and antibiotic resistance patterns and therefore should be carefully considered.

Significance and Impact of the Study: This study indicates that media and temperature can influence the recovery and selection of enterococcal species and antibiotic susceptibility.

Keywords: antibiotic resistance, enterococci, growth conditions, isolation, species.

INTRODUCTION

Collectively, members of the genus *Enterococcus* are characterized by their ability to hydrolyse esculin in the presence of bile salts and growth at temperatures between 10 and 45°C and in broth media containing 6·5% NaCl (Facklam and Collins 1989; Facklam *et al.* 2002). However, some species of enterococci are not representative of the entire group. *Enterococcus dispar* and *E. sulfureus* do not grow at 45°C, while *E. cecorum* and *E. columbae* do not grow at 10°C (Devriese *et al.* 1993). *Enterococcus avium*, *E. cecorum* and *E. columbae* may also exhibit no or poor growth in 6·5% NaCl broth medium. *Enterococcus cecorum* also does not grow in M-enterococcus media (Devriese *et al.* 1993). In addition, although enterococci can be routinely cultivated in the laboratory, they have been isolated from a number of different sources and are often found in populations of other microbial organisms creating problems for isolation (Domig *et al.* 2003).

Because of diverse sample types from which enterococci are isolated, more than 100 different modifications of selective media have been reported including various selective agents, indicators and cultivation temperatures (Reuter 1992; Domig *et al.* 2003). Although some methods have been well described, isolation of enterococci is complex not only due to the plethora of techniques available, but also due to the growth characteristics of some enterococcal species (Domig *et al.* 2003). Furthermore, studies on isolation of enterococci have been heavily based on enumeration,

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and the effect of the cultivation procedure on the species population of enterococci from samples has not been examined.

In this study, enterococci from poultry carcass rinsates were isolated using two commonly used selective media, Enterococcosel and M-enterococcus, two enrichment time periods, and three cultivation temperatures in order to evaluate the effect of these variables on the species population of enterococci.

MATERIALS AND METHODS

Bacterial isolation and species identification

All media used in this study were purchased from Becton Dickinson (Sparks, MD, USA). Enterococci in this study were isolated from 25 poultry carcass rinsates using three different enrichment temperatures (37, 42 and 45°C), two media types (Enterococcosel and M-enterococcus) and two incubation periods (24 and 48 h). Poultry carcass rinsates were collected from processing facilities in different regions of the USA, and rinsates from each carcass was processed separately to prevent cross-contamination. One millilitre aliquots of rinsates were inoculated into 9 ml BBL Enterococcosel broth and incubated for 24 h at 37°C, 42°C, or 45°C to enrich for enterococci. After 24 h, 100 μl of sample was inoculated onto M-enterococcus or Enterococcosel agar plates and incubated for 24 h at 37°C. Three single colonies of presumptive enterococcal isolates were subcultured onto slants of brain heart infusion agar (BHIA) for initial storage. For identification, single colonies were then subcultured twice onto BHIA plates or BHIA followed by blood agar (trypticase soy agar containing 5% defribinated sheep blood) for susceptibility testing. Enterococcal genus and species identification was performed as previously described (Jackson *et al.* 2004). A single colony of each positive culture was frozen in glycerol at −70°C. Following the initial 24-h incubation period, enrichment cultures were returned to the appropriate incubation temperature and allowed to continue incubating for an additional 24 h. Samples for the 48 h incubation temperature were processed as described above for the 24 h samples.

Antibiotic susceptibility testing

Minimum inhibitory concentrations (MIC; g l^{-1}) for enterococci were determined by broth microdilution using the Sensititre automated antibiotic susceptibility system (Trek Diagnostic Systems Limited, Westlake, OH, USA) according to the manufacturer's directions. Antibiotics and breakpoints were: bacitracin (≥128 g l^{-1}), chloramphenicol (≥32 g l^{-1}), ciprofloxacin (≥4 g l^{-1}), erythromycin (≥8 g l^{-1}), flavomycin (≥16 g l^{-1}), gentamicin (≥500 g l^{-1}), kanamycin (≥500 g l^{-1}), lincomycin (≥4 g l^{-1}), linezolid (≥8 g l^{-1}), nitrofurantoin (≥128 g l^{-1}), penicillin (≥16 g l^{-1}), salinomycin (≥16 g l^{-1}), streptomycin (≥1000 g l^{-1}), Synercid (≥4 g l^{-1}), tetracycline (≥16 g l^{-1}), tylosin (≥20 g l^{-1}) and vancomycin (≥32 g l^{-1}). Results were interpreted according to National Committee for Clinical Laboratory Standards (NCCLS) (2001) guidelines when defined. *Enterococcus faecalis* ATCC 29212 was the positive control for determination of MIC.

Statistical analysis

Probability values of statistical significance were generated using chi-square analysis. Statistical significance was defined as a probability value of ≤0·05.

RESULTS

Comparison of enrichment period, media and temperature on enterococcal species

A total of 817 enterococci were isolated from the study. Eighty-eight per cent (396 of 450) and 93·5% (421 of 450) of strains cultured from the 24 h and 48 h enrichments, respectively, were identified as enterococci (Table 1). Almost equal numbers of enterococci were isolated from Enterococcosel agar ($n = 412$) and M-enterococcus agar ($n = 405$). In contrast, although 91·7% (275 of 300) and 95·3% (286 of 300) of strains cultured at 37 and 42°C were identified as enterococci, only 85·5% (256 of 300) were identified as enterococci when cultured at 45°C (Table 1). At least 10 different species were identified from chicken carcass rinsates with *E. faecalis* ($n = 479$), *E. faecium* ($n = 185$) and *E. hirae* ($n = 52$) isolated most often (Table 1). Although no significant difference was observed among the species isolated for the 24- and 48-h enrichment periods, differences were found among species isolated on the different media types and at different incubation temperatures. On Enterococcosel agar, statistically less *E. faecalis* ($n = 226$, $P \leq 0{\cdot}025$) were isolated when compared with the number isolated from M-enterococcus agar ($n = 253$; Table 1). In contrast, more *E. faecium* ($P \leq 0{\cdot}01$) were isolated from Enterococcosel agar ($n = 111$) than from M-enterococcus agar ($n = 74$). No statistical difference for media was found among isolates of any other species.

Growth at various incubation temperatures affected the population of enterococci isolated in different ways. Three species appeared to be temperature-sensitive as the number isolated decreased as incubation temperature increased. More *E. faecalis* ($n = 188$) were isolated at 37°C than at 42°C ($n = 166$, $P \leq 0{\cdot}025$) and 45°C ($n = 125$, $P \leq 0{\cdot}001$; Table 1). The same pattern was seen for *E. casseliflavus* as more isolates were obtained at 37°C ($n = 10$) than at 42°C ($n = 0$, $P \leq 0{\cdot}01$) and 45°C ($n = 1$, $P \leq 0{\cdot}01$). In addition, more

Table 1 Comparison of isolated enterococcal species using various culture conditions

	Number of isolates (%)										
Culture condition	*E. faecalis*	*E. faecium*	*E. asini*	*E. avium*	*E. casseliflavus*	*E. durans*	*E. gallinarum*	*E. hirae*	*E. raffinosus*	*E. solitarius*	*Enterococcus* species
24 h enrichment ($n = 396$)	232 (58·6)	87 (22·0)	1 (0·3)	1 (0·3)	5 (1·3)	27 (6·8)	13 (3·3)	28 (7·1)	0	1 (0·3)	1 (0·3)
48 h enrichment ($n = 421$)	247 (58·7)	98 (23·2)	0	2 (0·5)	6 (1·4)	22 (5·2)	19 (4·5)	24 (5·7)	2 (0·5)	1 (0·2)	0
Enterococcosel agar ($n = 412$)	226 (54·9)	111 (26·9)	1 (0·2)	2 (0·5)	5 (1·2)	31 (7·5)	13 (3·2)	22 (5·3)	0	0	1 (0·2)
M-enterococcus agar ($n = 405$)	253 (62·5)	74 (18·3)	0	1 (0·2)	6 (1·5)	18 (4·4)	19 (4·7)	30 (7·4)	2 (0·5)	2 (0·5)	0
37°C ($n = 275$)	188 (68·4)	25 (9·1)	1 (0·4)	2 (0·7)	10 (3·6)	22 (8·0)	14 (5·1)	10 (3·6)	2 (0·7)	0	1 (0·4)
42°C ($n = 286$)	166 (58·0)	64 (22·4)	0	1 (0·3)	0	26 (9·1)	10 (3·5)	18 (6·3)	0	1 (0·3)	0
45°C ($n = 256$)	125 (48·8)	96 (37·5)	0	0	1 (0·4)	1 (0·4)	8 (3·1)	24 (9·4)	0	1 (0·4)	0

E. durans were also isolated at 37 ($n = 22$, $P \leq 0{\cdot}001$) and 42°C ($n = 26$, $P \leq 0{\cdot}001$) than at 45°C ($n = 1$). Conversely, two species, *E. faecium* and *E. hirae* appeared to be temperature-tolerant as numbers isolated increased as the temperature increased. Significantly more *E. faecium* ($P \leq 0{\cdot}001$) were isolated at 45°C than at 37 and 42°C, and more *E. hirae* ($P \leq 0{\cdot}01$) were isolated at 45°C than at 37°C (Table 1).

Effect of media and incubation temperature on antibiotic susceptibility

Antibiotic susceptibility was determined for *E. faecalis* and *E. faecium* isolates from Enterococcosel and M-enterococcus agar and at 37, 42 and 45°C. With the exception of four antibiotics (ciprofloxacin, linezolid, nitrofurantoin and Synercid), no differences were observed for *E. faecalis* or *E. faecium* isolates on the two media (Table 2). Significantly more ($P \leq 0{\cdot}001$) ciprofloxacin-, linezolid- and nitrofurantoin-resistant *E. faecalis* were isolated from Enterococcosel agar ($n = 18$, 12, and 16, respectively) compared with M-enterococcus medium ($n = 1$, 1, and 3, respectively; Table 2). In addition, more linezolid- and Synercid-resistant *E. faecium* ($P \leq 0{\cdot}05$) were also isolated from Enterococcosel agar ($n = 10$ and 91, respectively) than from M-enterococcus ($n = 1$ and 51, respectively).

With the exception of nitrofurantoin, none of the antibiotics which was affected by media was also affected by temperature. Differences in the number of antibiotic-resistant *E. faecalis* were found for bacitracin, flavomycin, gentamicin, nitrofurantoin, penicillin, streptomycin and tetracycline (Table 3). Statistically higher numbers of resistant *E. faecalis* were found for bacitracin ($P \leq 0{\cdot}025$) at 37°C ($n = 163$) than at 42°C ($n = 127$), and more gentamicin-resistant *E. faecalis* were isolated at 42°C ($n = 41$) than at 37°C ($n = 20$, $P \leq 0{\cdot}001$) or 45°C ($n = 19$, $P \leq 0{\cdot}05$). In contrast, more streptomycin-resistant *E. faecalis* were observed at 42 ($n = 40$, $P \leq 0{\cdot}025$) and 45°C ($n = 39$, $P \leq 0{\cdot}001$) than at 37°C ($n = 26$); while for tetracycline, statistically more resistant isolates were seen at 37 ($n = 148$, $P \leq 0{\cdot}01$) and 42°C ($n = 148$, $P \leq 0{\cdot}025$) than at 45°C ($n = 98$; Table 3). The same pattern of differences were seen for flavomycin ($P \leq 0{\cdot}001$ and $P \leq 0{\cdot}01$ for 37°C and 42°C, respectively), nitrofurantoin ($P \leq 0{\cdot}01$ and $P \leq 0{\cdot}05$ at 37°C and 42°C, respectively) and penicillin ($P \leq 0{\cdot}01$ for 37°C and 42°C) as higher numbers of resistant isolates were cultured at 45°C than any other temperature (Table 3).

For *E. faecium*, a distinction in the number of resistant isolates was seen for gentamicin, kanamycin, penicillin, streptomycin and tylosin (Table 3). The majority of differences for those antibiotics were at 45°C as higher numbers of resistant isolates were at this temperature. The number of gentamicin-resistant *E. faecium* increased as the temperature increased. More gentamicin-resistant isolates were obtained at 42°C ($n = 10$) than at 37°C ($n = 9$, $P \leq 0{\cdot}05$), but more were also obtained at 45°C ($n = 13$) than at 42°C ($P \leq 0{\cdot}05$; Table 3). For kanamycin ($P \leq 0{\cdot}01$) and penicillin ($P \leq 0{\cdot}01$), more resistant strains were isolated at 45°C ($n = 77$ and 60, respectively) than at 42°C ($n = 38$ and 25, respectively), while more streptomycin-resistant strains were cultured at 45°C ($n = 44$) than at both 37 ($n = 5$, $P \leq 0{\cdot}025$) and 42°C ($n = 17$, $P \leq 0{\cdot}025$). Tylosin-resistant *E. faecium* were more evident at higher temperatures (42 and 45°C) than at 37°C ($P \leq 0{\cdot}05$ and $P \leq 0{\cdot}01$ for 42°C and 45°C, respectively; Table 3).

DISCUSSION

From previous reports, the choice of which media to use for isolation of enterococci has primarily depended upon two

Table 2 Effect of media on antimicrobial resistance in enterococci

Species	Antimicrobial	Resistant, *n* (%)	
		Enterococcosel ($n = 226$)	M-enterococcus ($n = 253$)
Enterococcus faecalis	Bacitracin	181 (80·1)	214 (84·6)
	Chloramphenicol	12 (5·3)	8 (3·2)
	Ciprofloxacin	18 (8·0)	1 (0·4)
	Erythromycin	83 (36·7)	88 (34·8)
	Flavomycin	42 (18·6)	16 (6·3)
	Gentamicin	38 (16·8)	42 (16·6)
	Kanamycin	57 (25·2)	58 (22·9)
	Lincomycin	222 (98·2)	251 (99·2)
	Linezolid	12 (5·3)	1 (0·4)
	Nitrofurantoin	16 (7·1)	3 (1·2)
	Penicillin	5 (2·2)	3 (1·2)
	Salinomycin	3 (1·3)	0
	Streptomycin	47 (20·1)	58 (22·9)
	Synercid	212 (93·8)	245 (96·8)
	Tetracycline	185 (81·9)	209 (82·6)
	Tylosin	84 (37·2)	92 (36·4)
	Vancomycin	0	0
		Enterococcosel ($n = 111$)	M-enterococcus ($n = 74$)
Enterococcus faecium	Bacitracin	103 (92·8)	71 (96·0)
	Chloramphenicol	15 (13·5)	7 (9·5)
	Ciprofloxacin	23 (20·7)	8 (10·8)
	Erythromycin	54 (48·7)	41 (55·4)
	Flavomycin	84 (75·7)	59 (79·7)
	Gentamicin	24 (21·6)	8 (10·8)
	Kanamycin	82 (73·9)	50 (67·6)
	Lincomycin	106 (95·5)	71 (96·0)
	Linezolid	10 (9·0)	1 (1·4)
	Nitrofurantoin	43 (38·7)	35 (47·3)
	Penicillin	57 (51·4)	40 (54·1)
	Salinomycin	5 (4·5)	2 (2·7)
	Streptomycin	43 (38·7)	23 (31·1)
	Synercid	91 (82·0)	51 (68·9)
	Tetracycline	89 (80·2)	52 (70·3)
	Tylosin	51 (46·0)	39 (52·7)
	Vancomycin	0	0

goals: enumeration of enterococci or isolation from a highly contaminated source (Barnes 1976; Reuter 1985, 1992; Ford *et al.* 1994). M-enterococcus agar, also known as Slanetz-Bartley (SB) agar, is one of the oldest media formulations used for isolation and enumeration of enterococci mainly from water samples (Slanetz and Bartley 1957; Figueras *et al.* 1996). It contains 2,3,5-triphenyl tetrazolium chloride (TTC) and sodium azide as indicator and selective agents, respectively. In addition to its ability to distinguish enterococci from other bacteria, at pH 6·0, it can also distinguish *E. faecalis* from *E. faecium* as a result of the presence of TTC. Like M-enterococcus agar, Enterococcosel agar contains sodium azide but also contains bile and esculin as selective agents (Isenberg *et al.* 1970) and has been reported to be of particular use when samples are contaminated with other microbes (Domig *et al.* 2003).

Very few studies have addressed the impact of media on the selection of species population of enterococci. Reports on M-enterococcus agar indicate that growth of *E. cecorum* and *E. solitarius* is suppressed on this media (Devriese *et al.* 1993; Niemi and Ahtiainen 1995). No *E. cecorum* were isolated in this study, and although only two *E. solitarius* were identified, they were both from M-enterococcus agar. In this study, significant differences in the number of *E. faecalis* and *E. faecium* on M-enterococcus and Enterococcosel agar were found indicating that media can

Table 3 Effect of temperature on antimicrobial resistance in enterococci

Species	Antimicrobial	Resistant, *n* (%)		
		37°C ($n = 188$)	42°C ($n = 166$)	45°C ($n = 125$)
Enterococcus faecalis	Bacitracin	163 (86·7)	127 (76·5)	105 (84·0)
	Chloramphenicol	4 (2·1)	7 (4·2)	9 (7·2)
	Ciprofloxacin	3 (1·6)	8 (4·8)	8 (6·4)
	Erythromycin	68 (36·2)	57 (34·3)	46 (36·8)
	Flavomycin	14 (7·5)	16 (9·6)	28 (22·4)
	Gentamicin	20 (10·6)	41 (24·7)	19 (15·2)
	Kanamycin	36 (19·2)	49 (29·5)	30 (24·0)
	Lincomycin	185 (98·4)	165 (99·4)	123 (98·4)
	Linezolid	4 (2·1)	7 (4·2)	2 (1·6)
	Nitrofurantoin	3 (1·6)	5 (3·0)	11 (8·8)
	Penicillin	0	1 (0·6)	7 (5·6)
	Salinomycin	0	1 (0·6)	2 (1·6)
	Streptomycin	26 (13·8)	40 (24·1)	39 (31·2)
	Synercid	184 (97·9)	155 (93·4)	118 (94·4)
	Tetracycline	148 (78·7)	148 (89·2)	98 (78·4)
	Tylosin	72 (38·3)	58 (34·9)	46 (36·8)
	Vancomycin	0	0	0
		37°C ($n = 25$)	42°C ($n = 64$)	45°C ($n = 96$)
Enterococcus faecium	Bacitracin	24 (96·0)	62 (96·9)	88 (91·7)
	Chloramphenicol	1 (4·0)	6 (9·4)	15 (15·6)
	Ciprofloxacin	1 (4·0)	10 (15·6)	20 (20·8)
	Erythromycin	10 (40·0)	34 (53·1)	51 (53·1)
	Flavomycin	21 (84·0)	44 (68·8)	78 (81·3)
	Gentamicin	9 (36·0)	10 (15·6)	13 (13·5)
	Kanamycin	17 (68·0)	38 (59·4)	77 (80·2)
	Lincomycin	25 (100·0)	60 (93·8)	92 (95·8)
	Linezolid	2 (8·0)	5 (7·8)	4 (4·2)
	Nitrofurantoin	12 (48·0)	24 (37·5)	42 (43·8)
	Penicillin	12 (48·0)	25 (39·1)	60 (62·5)
	Salinomycin	1 (4·0)	2 (3·1)	4 (4·2)
	Streptomycin	5 (20·0)	17 (26·6)	44 (45·8)
	Synercid	20 (80·0)	50 (78·1)	72 (75·0)
	Tetracycline	21 (84·0)	50 (78·1)	70 (72·9)
	Tylosin	6 (24·0)	32 (50·0)	52 (54·2)
	Vancomycin	0	0	0

influence enterococcal species. Reasons for the differences are unknown, but could be due to specific media components because all other growth parameters were kept constant.

A number of studies have observed the differences in overall enterococci growth because of varying cultivation temperature (Dutka and Kwan 1978; Peterz and Steneryd 1993; Niemi and Niemela 1994; Devriese *et al.* 1995; Niemi and Ahtiainen 1995). Increased cultivation temperatures (up to 45°C) decreased the contaminating growth in numerous types of samples including water and dairy samples. Peterz and Steneryd (1993) reported an increase in the number of enterococci-positive colonies at 44°C compared with 37°C, attributing the results to the higher amount of contaminating organisms and a higher percentage of false-positives at 37°C *vs* 44°C. Furthermore, the species of enterococci were not reported, only bacteria identified as the genus enterococci. Opposite results were reported in another study which found that growth of faecal streptococci (enterococci) was inhibited at elevated temperature (44·5°C) when combined with M-enterococcus media (Dutka and Kwan 1978).

One limitation with elevated temperature is decreased numbers of enterococci possibly due to the species of enterococci that are temperature-sensitive. In one study, *E. durans* appeared to be more temperature-sensitive than other enterococcal strains (Niemi and Ahtiainen 1995). Results from the same study indicated that isolation of *E. faecalis* and *E. hirae* was also improved at 41°C rather

than 44°C, but the results were not significantly different at the two temperatures. In this study, significant differences were found in the prevalence of *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans* and *E. casseliflavus* at the three temperatures tested. *Enterococcus faecalis*, *E. durans* and *E. casseliflavus* appeared to be temperature-sensitive while *E. faecium* and *E. hirae* were temperature-tolerant. Variation in temperature tolerance appears to be an inherent property of different enterococcal species as some will not grow at the lower and higher ranges of temperature (10–45°C) described for the genus *Enterococcus* (Devriese *et al.* 1993; Deasy *et al.* 2000).

To our knowledge, this is the first study noting antibiotic resistance differences of enterococci grown in different media or at different temperatures. Other studies have documented differences in antibiotic resistance when various chemicals were added to the susceptibility testing media or when different incubation temperatures were used during susceptibility testing (Dutta and Devriese 1982; Butaye *et al.* 1998, 2000). These manipulations were not used in this study. For media, in each case where there was an increased incidence of resistant isolates, they were all from Enterococcosel agar. This finding suggests that the differences were not due to the number of isolates recovered because higher numbers of *E. faecalis* were isolated from M-enterococcus agar than from Enterococcosel. Both media and temperature could have caused physiological changes in the bacteria resulting in increased or decreased resistance. Common antibiotic resistance affected were linezolid for media and gentamicin, penicillin and streptomycin for temperature regardless of species (*E. faecalis* or *E. faecium*). Additional studies on this phenomenon will need to be conducted in order to determine factors behind the effects of media and temperature variations.

In conclusion, for population studies on enterococci from any source, particular attention should be directed to methods of cultivation in order to obtain accurate data.

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